APPENDIX A

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Biochemistry

Crystal structure of the catalytic domain of *Pseudomonas* exotoxin A complexed with a nicotinamide adenine dinucleotide analog: Implications for the activation process and for ADP ribosylation

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ABSTRACT **The catalytic, or third domain of *Pseudomonas* exotoxin A (PEIII) catalyzes the transfer of ADP ribose from nicotinamide adenine dinucleotide (NAD) to elongation factor-2 in eukaryotic cells, inhibiting protein synthesis. We have determined the structure of PEIII crystallized in the presence of NAD to define the site of binding and mechanism of activation. However, NAD undergoes a slow hydrolysis and the crystal structure revealed only the hydrolysis products, AMP and nicotinamide, bound to the enzyme. To better define the site of NAD binding, we have now crystallized PEIII in the presence of a less hydrolyzable NAD analog, β-methylene-thiazole-4-carboxamide adenine dinucleotide (β-TAD), and refined the complex structure at 2.3 Å resolution. There are two independent molecules of PEIII in the crystal, and the conformations of β-TAD show some differences in the two binding sites. The β-TAD attached to molecule 2 appears to have been hydrolyzed between the pyrophosphate and the nicotinamide ribose. However, molecule 1 binds to an intact β-TAD and has no crystal packing contacts in the vicinity of the binding site, so that the observed conformation and interaction with the PEIII most likely resembles that of NAD bound to PEIII in solution. We have compared this complex with the catalytic domains of diphtheria toxin, heat labile enterotoxin, and pertussis toxin, all three of which it closely resembles.**

Pseudomonas exotoxin A (PE) belongs to a family of bacterial nicotinamide adenine dinucleotide (NAD)-binding toxins that ADP ribosylate target proteins within eukaryotic cells (1). Crystal structures have been reported for four of these toxins: PE (2), diphtheria toxin (DT) (3), *Escherichia coli* heat-labile enterotoxin (LT) (4), and pertussis toxin (PT) (5), revealing a common fold for the catalytic ADP ribosylating domain, consisting of two approximately orthogonal, antiparallel β sheets flanked by several helices forming a large substrate binding cleft at the center of the domain. The folding topology is unlike the dinucleotide binding fold observed in several dehydrogenases (6).

PE is a 613-amino acids protein that is composed of three structural domains. Domain I (1–252 and 365–399) binds to the ubiquitous α2-macroglobulin receptor of eukaryotic cells and initiates receptor-mediated endocytosis (7). In the cell, PE is cleaved at residue 280 within domain II (residues 253 to 364) by a specific protease (8); this cleavage activates the third domain. The portion of domain II (residues 280–364) remaining with the C- terminal fragment appears to translocate the fragment through intracellular membranes into the cytosol, where domain III (residues 400–613) acts by transferring the ADP ribose from NAD to a modified histidine in elongation factor-2 (EF-2). This irreversible covalent modification inhibits protein synthesis and leads to cell death. The function of domain Ib (residues 365–399) is unknown and it can be entirely deleted without loss of toxin activity (9).

In vitro, the intact toxin molecule has low affinity for NAD and exhibits no ADP ribosyl transferase activity (2). Treatment with reducing and denaturing agents is necessary to obtain an active molecule that can both bind to NAD and effect ADP ribosylation of EF-2 (10). The isolated domain III (PEIII) is active in the absence of this activation treatment (11). PEIII also exhibits a weak NAD glycohydrolase activity (12). We have determined (13) the structure of this catalytic domain complexed with the products of NAD hydrolysis, AMP and nicotinamide, thus effectively defining the active site. This structure revealed that the process of activation must involve the removal of helix 333 to 353 of domain II from the vicinity of the active site loop 458 to 463 of domain III, thus permitting a conformational change in this loop that is necessary for NAD binding.

In this structure, although the crystals were grown in the presence of NAD, resulting hydrolysis prevented the visualization of intact NAD in the binding site. We observed density corresponding to AMP and nicotinamide, the apparent final products of the hydrolysis, in a cleft containing residues that have been identified by mutagenesis as binding to NAD (10, 14). The locations define the ends of the NAD, but the flexibility in the diphosphate backbone and uncertainty in the orientations of the ribose rings did not permit unambiguous modeling of the intact NAD. In this work, we describe the refined structure of PEIII complexed with a less hydrolyzable NAD analog, β-methylene-thiazole-4-carboxamide adenine dinucleotide (β-TAD). In this analog, a five-membered thiazole ring replaces nicotinamide, a C-glycosidic bond replaces an N-glycosidic bond and a carbon replaces the β-oxygen in the pyrophosphate. These substitutions render the compound resistant to hydrolysis in a structure that is quite similar to NAD. This allows us for the first time to describe the interactions between intact NAD and PEIII that have important implications for the mechanism of the ADP ribosyl transfer. Recently, the structure of the DT catalytic domain has been reported complexed with NAD, which was trapped by freezing prior to hydrolysis (15).

EXPERIMENTAL PROCEDURES

The PEIII was expressed in IPTG-induced cultures of *Escherichia coli* BL21(λDE3) carrying plasmid PEΔ5–399. TAD

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Abbreviations: PE, *Pseudomonas* exotoxin A; PEIII, the catalytic domain of PE; β-TAD, β-methylene-thiazole-4-carboxamide adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; EF-2, elongation factor-2; DT, diphtheria toxin; LT, heat-labile enterotoxin from *E. coli*; PT, pertussis toxin.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1AER).

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was provided by V. E. Marquez (National Institutes of Health). Cocrystallization of PEIII and TAD was conducted using the hanging drop technique. Crystals were obtained under the same conditions as those of PEIII with NAD and were isomorphous with those crystals (13). X-ray diffraction data were collected on an R-AXIS-II imaging plate detector Molecular Structure, The Woodlands, TX at room temperature and processed using the DENZO and SCALEPACK provided by Z. Otwinowski (University of Texas, Dallas, TX). The structure was refined using X-PLOR version 3.1 (16) against 2.3 Å data to an R-factor of 19.6% (for data greater than 2σ between 8.0 and 2.3 Å), with good stereochemistry (Table 1).

Crystals of PEIII/β-TAD were transferred to a well containing solution of 1.5 M sodium citrate in 0.1 M Tris (pH 7.8). The well solution was exchanged every hour during the first day and once a day afterwards for about a month. The X-ray diffraction data of 2.8 Å were collected under the same conditions as data of PEIII/β-TAD crystals. Data were indexed and integrated with DENZO and scaled with SCALEPACK. The soaked crystals are isomorphous with those complexed with β-TAD. The refinement was conducted using X-PLOR with noncrystallographic 2-fold axis restraints (16). The statistics of data and results of refinement are listed in Table 1.

RESULTS

The crystals of the complex of PEIII with β-TAD are isomorphous with those previously obtained from PEIII with hydrolyzed NAD, with two molecules in the asymmetric unit. The two protein molecules adopt similar conformations and they bind β-TAD in similar ways, but because of different crystal contacts, they are not identical (Fig. 1 *A* and *B*). In both molecules the adenine forms hydrogen bonds with the carbonyl oxygen of Gly-454, the amide nitrogen of Arg-456 and the side chain of Ser-449. The adjacent ribose hydrogen bonds to His-440 and Thr-442. These interactions are very similar to those observed between PEIII and AMP (13). The thiazole ring stacks against Tyr-481 and forms hydrogen bonds with the amide nitrogen and the carbonyl oxygen of Gly-441 in a manner quite similar to the nicotinamide in the previous structure (13). Although there is an overall similarity between the binding sites of the two crystallographically distinct molecules, there are also significant differences. One of these occurs in the loop between residues 457 and 464 in molecule 1, where the electron density is weak and uninterpretable, suggesting local disorder. In molecule 2, this loop assumes a more stable conformation, perhaps because of crystal packing interactions; three residues, Arg-458, Ser-459, and Asp-461 make hydrogen bonds with a neighboring molecule, and the O5′ of the adenosine of β-TAD also bonds to the Arg-609 of a symmetry related molecule. In molecule 1 there is a hydrogen bond between Glu-553 and the O2′ of the thiazole ribose; this bond is not observed in molecule 2.

The conformations of β-TAD in the two molecules are also different. In particular, there is a break in the electron density of the β-TAD between C5′ of the thiazole ribose and O5′ of the second phosphate of molecule 2 indicating that hydrolysis has occurred in this region. (Fig. 2). This is supported by the observation that when the crystallographic refinement was repeated with a broken bond in this position there was a 0.3% drop of the Rfree crossvalidated crystallographic residual (17). These differences are probably the result of the different packing environments around the two binding sites. In molecule 2, Arg-609 from a symmetry-related molecule stacks against the adenine and also hydrogen bonds to the AMP phosphate. This packing interaction perturbs the β-TAD binding and could be responsible for the hydrolysis in molecule 2 that is not observed in molecule 1.

The configuration of the β-TAD observed in molecule 1 (Fig. 1) is likely to be very similar to the conformation of NAD bound to the isolated PEIII in solution. The analog β-TAD differs from NAD in two respects: (*i*) the replacement of the positively charged nicotinamide by a neutral β-thiazole-4-carboxamide ring system, where the amide group of both systems bonds to the carbonyl oxygen and NH of Gly-441, and (*ii*) the replacement of a β-oxygen of the pyrophosphate by a CH_2. Neither of these changes are likely to result in major differences in the binding conformation. We have accordingly modeled the NAD in the binding site of the PEIII monomer 1, replacing the β-methylene group with an oxygen and the thiazole with nicotinamide.

The AMP conformation is unusual in that the ribose adopts the seldom observed "high syn" conformation ($\chi = 95°$) relative to the adenine (18). For the nicotinamide or the thiazole rings "syn" and "anti" has less significance in the absence of a substituent on either side of the glycosidic bond; the observed $\chi = -21°$ (O4′—C1′—C2—N3) corresponds to syn relative to the N3 position of the thiazole ring. The sugar puckers correspond to C2′—endo for AMP and C3′—endo for the thiazole ribose. The γ angle, O5′—C5′—C4′—C3′ at the AMP is −68°. The general disposition of the NAD presents the pyrophosphate backbone on the outside of the active site cleft. Similarly, the scissile C1′—N bond is on the outside of the nicotinamide ring that points back into the cleft. It should be noted that the conformation observed here for β-TAD is quite different from the conformation adopted when bound to alcohol dehydrogenase (19), where the glycosidic angles (χ) at both the adenosine and the thiazole-ribose are different.

In those crystals from which the β-TAD was removed by washing, the structure of the PEIII remained essentially the same with weak uninterpretable density for the loop region 457–464 of molecule 1 and well defined ordered density in molecule 2.

Table 1. Summary of crystallographic refinement

	PEIII/β-TAD	Free PEIII
Data collection statistics		
Space	$P4_32_12$	$P4_32_12$
Cell Parameters		
a	87.64Å	87.33Å
c	133.73Å	132.90Å
Molecules/asymm. unit	2	2
Resolution (Å)	2.3	2.8
Unique reflections	22417	12147
Completeness (%)	93.4	90.4
Shell 2.4–2.3 Å* and 2.9–2.8Å†	78.4	87.4
R_{sym} (%)	5.7	8.8 (I>0)
Average redundancy	4.7	3.2
Refinement results		
Resolution	8.0–2.3	10.0–2.8
Atoms (no.)	3216	3003
Water (no.)	110	none
Rfree (%)	28.5	32.0
R (%)	19.6	20.9
RMS Δ Bond lengths (Å)	0.012	0.012
RMS Δ Bond angles (deg.)	2.048	2.369

*PEIII/β-TAD.
†Free PEIII.

DISCUSSION

Comparison with Intact PE. The only significant conformational change observed between intact PE and the β-TAD bound form of PEIII is in the loop between residues 457–464, adjacent to the active site. In molecule 1 this loop is disordered and does not appear to be directly involved in NAD binding. In monomer 2 it is ordered, but in a conformation that is significantly different from that in the intact PE crystal

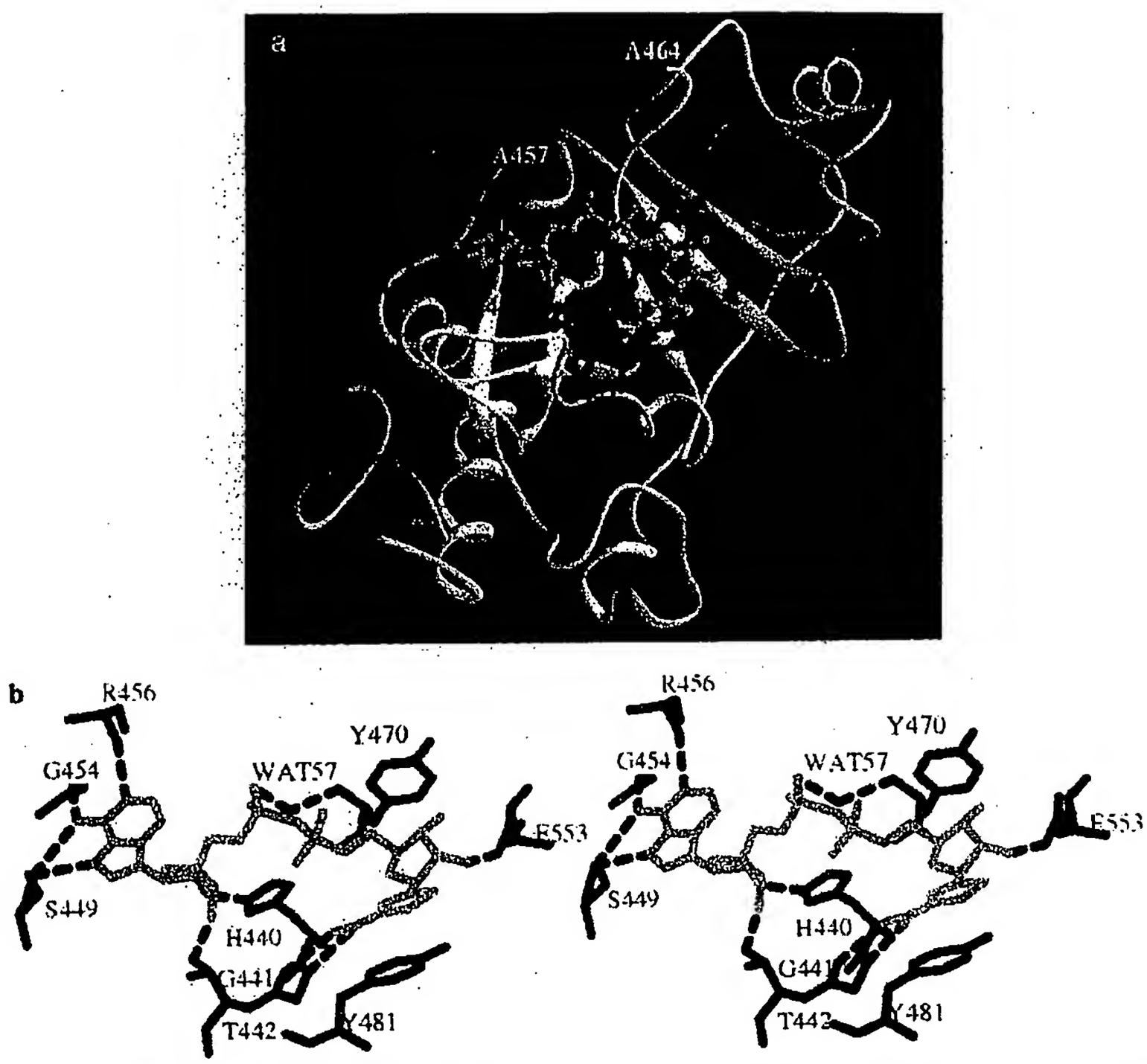

FIG. 1. (*a*) The binding of β-TAD (yellow) in the active site of molecule 1 (white), showing the residues involved in binding (red). The loop connecting residues 457 and 464 is disordered. (*b*) Stereoview of the hydrogen bonds formed between the β-TAD and the surrounding residues.

structure with a rms difference in Cα positions of 7.6 Å for residues 458–463. These results support the observations made previously for the isomorphous crystal of PEIII complexed with NAD hydrolysis products (13).

The binding mode for NAD observed here would produce unacceptably close contacts if the loop between residues 456 and 463 adopted the conformation seen in the intact PE structure (Fig. 3). These contacts involve the side chains of Arg-458 and Gln-460 and probably account for the observed very weak binding of NAD to intact PE (2). Movement of this loop away from the binding site is necessary for the substrate to bind freely and is presumably part of the activation process. However, in intact PE this movement is prevented by helix 333 to 355 of domain II. The action of reducing and denaturing agents in activating PE probably occurs by disrupting the structure of domains Ib and II, with reduction of the disulfide bonds between Cys-265 and Cys-287, and between Cys-372 and Cys-379 (12). The *in vivo* activation by cleavage at Gly-280 results in a structure that is at present unknown, but the activation here also must involve movement of the active site loop.

We attempted to determine the effect of β-TAD binding on the disordered loop, but attempts to determine the shape of the active site in the absence of NAD were complicated by the failure to grow crystals in the absence of either NAD or β-TAD. This growth failure necessitated the removal of the NAD analog after the crystals had grown. Accordingly, we soaked the crystals of PEIII/β-TAD extensively in crystallizing solution that did not contain β-TAD. The refined electron density for molecule 1 indicates that the β-TAD has been removed from the active site. However, the PEIII structure is unchanged and there is a continued absence of density for the loop 458–463, implying that the binding of NAD does not cause conformational changes in PEIII and does not stabilize the conformation of this loop. For the molecule 2 there is still density in the active site corresponding to the continued binding of AMP, although there is no density for the thiazole and associated ribose, and here the loop 458–463 remains visible.

Implications for ADP ribosylation. The formation of a binary complex between NAD and PEIII is a prerequisite for the binding of PEIII to EF-2 to occur (20). The above results indicate that no changes are observed in PEIII upon NAD binding. However, the location of NAD on the surface of molecule I with solvent-exposed phosphate groups permits a direct interaction between the NAD and EF-2.

Since DT also ADP ribosylates EF-2, we have made a three-dimensional alignment of the PEIII β-TAD (molecule 2), in the vicinity of active site region. A comparison of 30 Cα positions (rms = 1.34 Å reveals close structural homology, with residues Asp-463, Trp-466, and Arg-467 in PEIII corresponding to Asp-47, Trp-50, and Lys-51 in DT. None of these residues are directly involved in NAD binding. They all are accessible to the solvent and they, perhaps together with the shifting loop residues, could play a role in the interaction between the PEIII-NAD binary complex and EF-2.

The catalytic process of ADP ribosylation has been shown to involve residues His-440 and Glu-553 (21, 22). Substitution of Ala, Asn, and Phe for His440 severely reduces the ADP ribosylation activity without diminishing the glycohydrolase activity (21). In the β-TAD complex with the isolated domain III, the side chain of His-440 is located in the vicinity of the

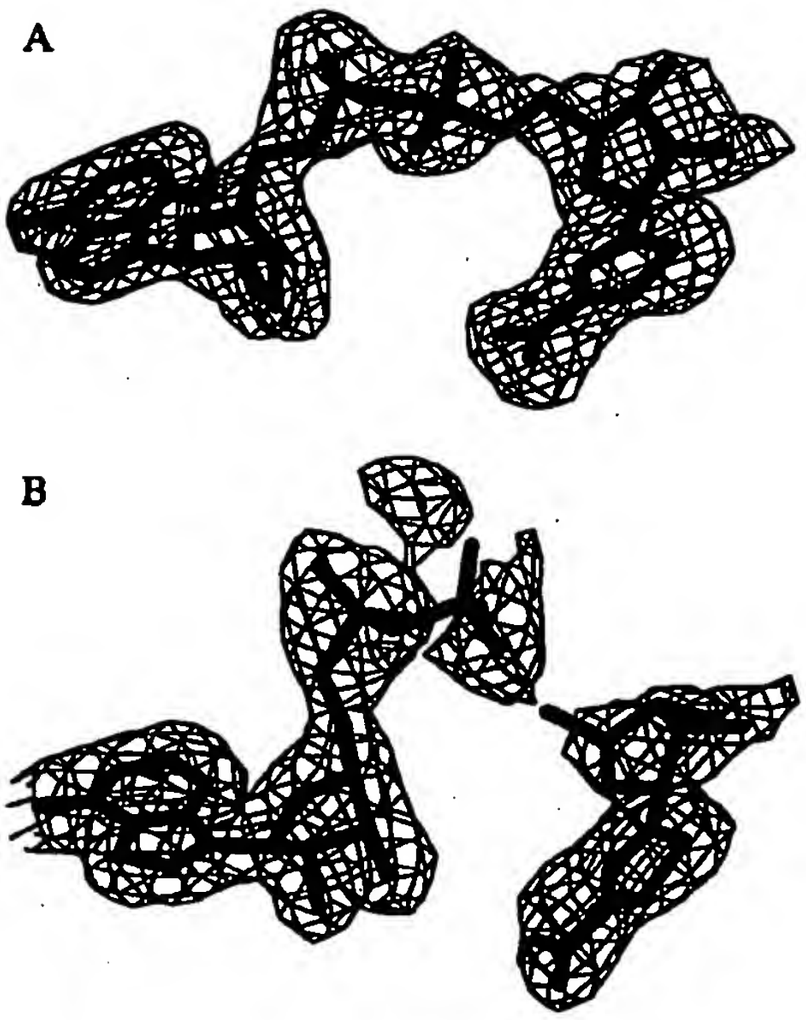

FIG. 2. 2Fo-Fc map of (*a*) β-TAD bound to molecule 1. (*b*) Hydrolysis products of β-TAD on molecule 2 contoured at 1σ.

AMP ribose where it forms a hydrogen bond with O2′ of the ribose and also with the main chain carbonyl of Tyr-470, a residue involved in van-der-Waals interactions with the thiazole-ribose moiety of β-TAD. In this structure His440 plays a role in binding the NAD, but it seems to be too distant from the scissile glycosidic bond of the ribose to play a direct role in catalysis, consistent with the observations of weak ADP ribosylation activity by PE when this residue was replaced (21).

Substitution of Glu-553 with Asp caused a 3200-fold reduction in the ADP ribosyltransferase activity of PE (23), but caused only minor reductions in substrate affinity and on the NAD-glycohydrolase activity (23, 24). Replacement of Glu-553 with the uncharged Gln also diminished the ADP ribosyltransferase activity, suggesting that a carboxyl group is needed for catalysis (24). A photoproduct of C6 of nicotinamide covalently linked to the γ-methylene group of Glu-553 was detected in photolabeling experiments (22). In this structure Glu-553 forms a hydrogen bond with O2′ of the thiazole ribose (2.4 Å). Here it can play an important role in helping to maintain the NAD in a conformation that exposes C1′ to the attacking diphthamide while leaving sufficient room for the reaction to proceed with an inversion of configuration at C1′ (25). In addition, OE2 of the carboxylate group is within 4.0 Å of the thiazole-ribose bond, its negative charge therefore is available to stabilize a positively charged intermediate. The role of Glu-553 in NAD binding is probably further facilitated by its involvement in a water-mediated hydrogen bond (together with Glu-546) with the hydroxyl group of Tyr-481, a residue that forms a ring stacking interaction with the nicotinamide (13).

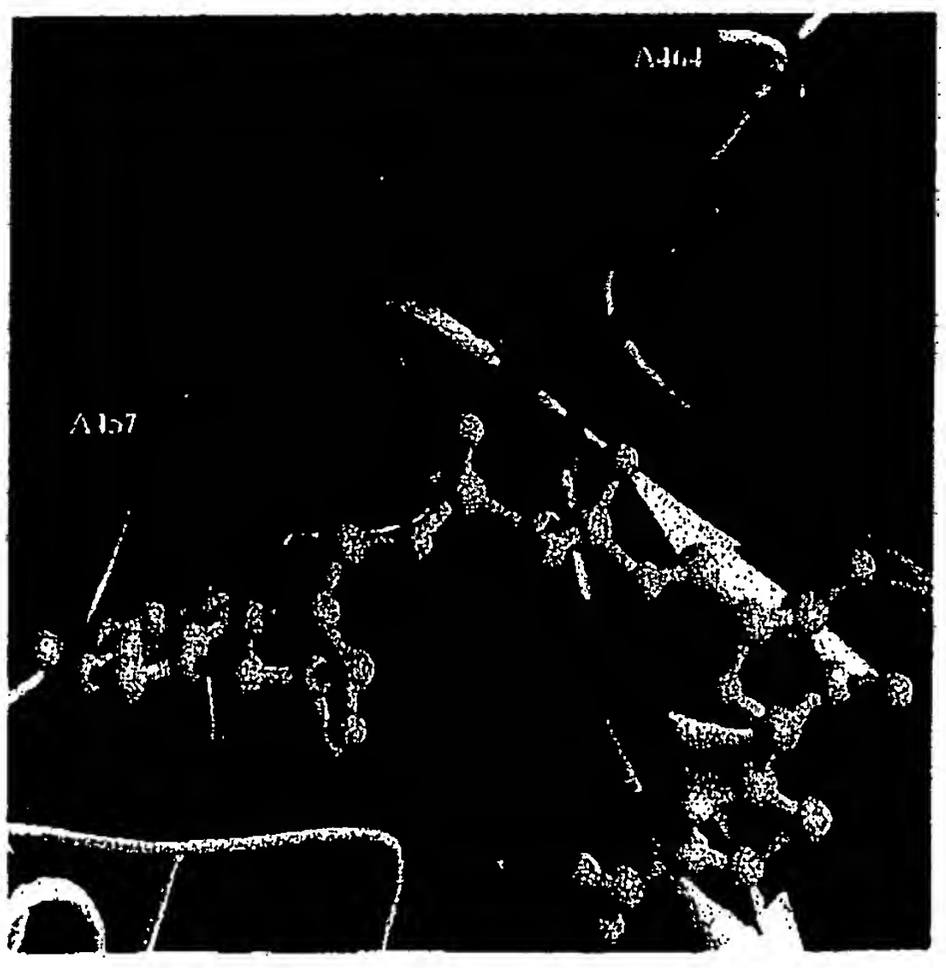

FIG. 3. The superposition of the β-TAD binding site for domain III of intact PE (magenta), PEIII with and without β-TAD (white and green, respectively). For domain III of intact PE the side chains of the flexible loop have been included to show the close contacts with the β-TAD.

Comparison with DT and Other Toxins. There has been a recent report of the structure of NAD complexed with intact DT at low temperature (15). The amino acid residues that interact with the NAD are strikingly similar to those observed in this β-TAD complex and for the hydrolysis products of NAD bound to PEIII (13). Bell and Eisenberg (15) raise the question of whether the structure they observe is biologically relevant since it is the C-domain alone that acts on EF-2 and *in vitro* studies have shown that whole DT does not possess detectable ADP ribosylation activity against EF-2. The results presented here involve domain III, the catalytically active form of PE (27, 28), and the similarity of the nucleotide binding sites in both PEIII and DT, including the disorder in the flexible loop, establishes that this is the catalytically active form used to inactivate EF-2.

Superposition of 46 residues (5) from the PEIII on the equivalent atoms in three other toxins, DT, LT, and PT, reveals the similarity of these active sites. However, the flexible loop region corresponding to 458 to 463 in PEIII does not show similarity with the other toxins. The lengths of the loops are different, from 38–47 in DT, from 25–57 in LT (4, 29) and from A26 to A47 in PT. The β-TAD can be placed in the active site of DT without forming unacceptable contacts with this loop. In LT the β-TAD forms unacceptable close contacts with the loop, 47 to 56. In PT it does not collide with the loop but it does collide with helix 199–206, located within the active site of the intact PT structure. These different loop lengths and interactions with the NAD may be a reflection of different activation processes and different target proteins for LT and PT (4, 5).

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